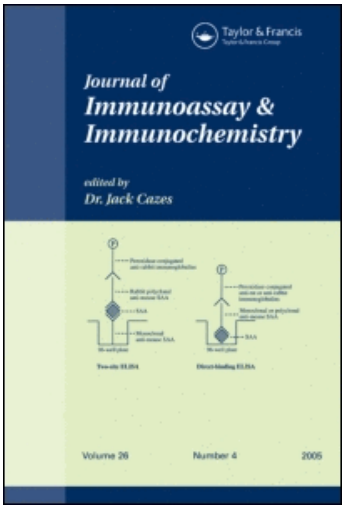

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Development and Validation of a Sensitive Radioimmunoassay for Progesterone Estimation in Unextracted Mithun (*Bos frontalis*) Plasma

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Development and Validation of a Sensitive Radioimmunoassay for Progesterone Estimation in Unextracted Mithun (*Bos frontalis*) Plasma

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Abstract: The objective of the study was to develop and validate a simple, reliable, and highly sensitive radioimmunoassay (RIA) for progesterone determination in mithun (*Bos frontalis*) plasma. The RIA was carried out in 20 μL unextracted mithun plasma. The progesterone standards ranging from 2 to 500 pg/20 μL/tube were prepared in charcoal-treated hormone-free plasma. The sensitivity of the RIA procedure was 2 pg progesterone/20 μL/tube, which corresponds to 0.1 ng/mL; the 50 percent relative binding sensitivity was seen at 32 pg/20 μL/tube. Plasma volumes for the RIA, viz. 10 and 20 μL, did not influence the shape of standard curve, even though a slight drop in the counts was seen with higher plasma volumes. For the biological validation of the assay, three cyclic, three in early pregnancy, and two in late pregnancy mithuns were used. Blood samples collected at weekly intervals for 42 days, from all animals, were assayed for plasma progesterone. The peak level of progesterone was registered at day 14 (day 21 of sampling) of the estrous cycle and the lowest at estrus; the progesterone concentrations increased and decreased gradually as sampling time advanced, in early and late pregnancy, respectively, which confirm the biological validation of the RIA. The RIA avoids the troublesome and laborious plasma extraction procedures. In conclusion, the RIA developed for progesterone determination in mithun blood plasma is sufficiently

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reliable, simple, and sensitive enough to estimate progesterone in all physiological variations in mithun.

Keywords: Mithun-endocrinology, Progesterone, RIA, Validation, Plasma, Semi-wild

INTRODUCTION

Mithun (*Bos frontalis*), which is believed to originate more than 8000 years ago, is considered to be descended from wild gaur.[1] This animal is a semi-wild ruminant species, found mainly in the North-Eastern Hills region (NEHR) of India. This unique livestock species is also found, though lower in population, in Bhutan, Myanmar, Bangladesh, China, and Malaysia. This prized hill animal, NEHR, has an important role in the economic, social, cultural, and religious life of the local tribal population under the undulating topography and adverse climatic conditions at moderately high altitudes (300 to 3000 m above mean sea level). The multifarious utility of mithun is well recognized. It acts as a potential source of meat. Growth rate of this animal is comparable with that of cattle or buffalo under adequate nutrition.[2] Recently, the milk production potential of mithun has also been explored with the ability to produce superior quality milk. The age at puberty varies between 22 to 30 months (26 ± 4 months) and body weight is around 250 kg. Due to remoteness of their habitats and other ecological and socio-political factors, mithuns remain among the most neglected ungulates.

The estimation of progesterone in blood has found practical application as a tool for improving reproduction in farm animals. Blood progesterone can provide a true picture of ovarian activity and, thereby, the reproductive status of the animal. Progesterone has been measured for the purpose of pregnancy diagnosis in cattle,[3–5] pigs,[6,7] buffaloes,[8] goats,[9] for confirmation of estrus in cattle[4,5] and buffaloes[8] and for monitoring fertility in cattle.[5,10,11] Confirmation of age at sexual maturity is also practiced by estimating plasma progesterone in different species of animals.[12–14]

To conduct further research in these fields on mithun, a reliable and sensitive assay procedure for progesterone estimation is needed. No assay procedure has so far been developed for measurement of progesterone in mithun blood plasma. The present study was, therefore, designed to develop a sensitive radioimmunoassay (RIA) for progesterone in unextracted mithun plasma.

EXPERIMENTALS

Materials for RIA

Radiochemicals

The radiochemical (tracer) in the assay namely (1,2,6,7,16-^{3}H)-progesterone (93.0 μCi/mmol) was purchased from M/s Radio Chemical Centre, Amersham, England.

Chemicals (Non-Radioactive)

Non-radioactive chemicals used in this investigation were either imported from M/s Sigma-Aldrich Chemie, St. Louis, USA or purchased locally from reputable firms. The chemicals obtained from abroad include chemicals such as 2,5-diphenyloxazole (PPO), 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP), dextran (molecular weight 70,000), and thiomersal. The chemicals obtained locally were sodium chloride, sodium dihydrogen phosphate [NaH_2PO_4, $2H_2O$], sodium phosphate dibasic [Na_2HPO_4], BSA (all used for preparation of phosphate buffer saline) and toluene (for use in scintillation cocktail).

Progesterone Antibody

The progesterone antibody used in the present study [BSP NR#2 (20.10.93)] was highly specific for progesterone and was raised at National Dairy Research Institute, India.[15] Briefly, about 300 μL of progesterone-α-OH-hemisuccinate-BSA (3 mg) dissolved in phosphate buffered saline ($NaPO_4$ 0.04 M, NaCl 0.15 M; 1 mg/mL), emulsified with an equal volume of Freund's complete adjuvant (1 : 1) was administered to four male Soviet Chinchilla rabbits intradermally at 30–40 sites on the flank regions. Six booster immunizations with the same amount were administered in similar fashion at intervals of one to one and half months. Blood samples (2 mL) were collected in clean test tubes 2 days after booster administration, and at weekly intervals, from the immunized rabbits. The blood samples were allowed to clot at room temperature for 30 min and serum was separated by centrifugation. As provided by Prakash and Madan,[15] the cross reactivity of the progesterone antibody with corticosterone was <0.20 and with all other steroids tested was <0.001 percent.

Preparation of Progesterone Free Plasma

The plasma obtained from blood samples collected at day 3 of parturition, when plasma progesterone concentration is very low, were treated as follows to get progesterone free plasma:

1. To 50 mL plasma, 10 g of activated charcoal and 1 g of Dextran T-70 in 100 mL of normal saline were added in a beaker and the contents were stirred with a magnetic stirrer overnight at 4°C. Thereafter, the supernatant was collected after centrifugation at 2000 × g for 30 minutes.
2. The above procedure was repeated twice.
3. The supernatant was then passed through 1, 0.45, and 0.22 μm filters. The filtered plasma was stored in 2 mL-leveled vials at −20°C after the test for hormone free plasma.

Test for Charcoal Treated Plasma

Two sets of triplicate RIA tubes containing 300 μL assay buffer ($NaPO_4$ 50 mM, NaCl 0.15 M, thimerosal 0.02%: pH 7.2), 100 μL diluted progesterone specific antisera (1 : 8000), 100 μL tracer (8500 counts), and either 20 μL assay buffer or 20 μL charcoal treated plasma were run in an assay following the rest of the procedure as described under assay procedure section below. The mean counts between the tube sets was not different ($P > 0.05$; 1359 ± 35 and 1344 ± 40 counts for the RIA sets containing 20 μL assay buffer and 20 μL charcoal treated plasma, respectively) confirming that the charcoal treated plasma was progesterone free.

Assay Procedure

Duplicates of 20 μL plasma samples were taken in 12 × 100 mm test tubes along with the addition of PBS buffer (Na_3PO_4 50 mM, NaCl 0.15 M, thimerosal 0.02%, pH 7.2) to make the volume up to 320 μL. Thereafter, 0.1 mL of progesterone specific antiserum was added (1 : 8000) to each tube followed by addition of 0.1 mL of tracer (8500 CPM). All tubes were then incubated at room temperature overnight for the immune reaction. Free and antiserum bound hormone were separated by addition of a freshly prepared cold charcoal-dextran suspension (1% activated charcoal plus 0.1% dextran in buffer). The tubes were then stirred, incubated at 4°C in ice water bath for 15 min, and then centrifuged at 3000 rpm at 4°C for 15 min. The supernatant containing the bound progesterone was decanted into scintillation vials and counted in 5 mL of scintillation fluid (0.1 g POPOP and 4 g PPO in 1000 mL of scintillation grade toluene) using a liquid scintillation counter (Model: LS 6000, Beckman, USA) for two minutes. Along with the samples, standard progesterone samples in charcoal-treated hormone-free plasma were also run in duplicate, ranging from 2 to 500 pg/20 μL.

In addition to the above, three sets of tubes were also run as follows:

a) *Non-specific binding tubes* (blank tubes), in duplicate, containing 400 μL of assay buffer (PBS plus 0.12% gelatin), 20 μL of charcoal-treated plasma and 100 μL of tracer, to observe non-specific binding in the assay.
b) *Maximum binding tubes*, four tubes containing 300 μL assay buffer, 20 μL of charcoal-treated plasma, 100 μL antiserum and 100 μL tracer to obtain maximum binding of tracer by the antisera used in the assay.
c) *Total count tubes*, in duplicate containing 100 μL tracer diluted with 900 μL assay buffer and 20 μL of charcoal-treated plasma to obtain total counts of tracer added and decanted directly into the scintillation vials.

The average counts of non-specific binding tubes (error or background counts) were subtracted from counts of all samples as well as standards.

Biological Validation of the Mithun Plasma Progesterone RIA

For the biological validation of the assay, three cyclic, three in early pregnancy, and two in late pregnancy mithuns were used. Blood samples (3.5 mL) were collected by jugular venipuncture in heparinized tubes (20 IU heparin sodium/mL) from all animals at weekly intervals for 42 days. All blood samples were centrifuged at 3000 rpm for 30 min at 4°C and plasma separated out was stored at −20°C till analyzed for progesterone. All experimental protocols and animal care met the regulations of Institutional Animal Care and Use Committee (IACUC).

Calculation of Intra- and Inter-Assay Precision

Duplicate tubes containing known concentrations of low (0.8 ng/mL) and high (12.5 ng/mL) progesterone were run in three places in each assay. Percent of coefficient of variation (CV) was then calculated for each (intra-assay CV) and among different assays (inter-assay CV) using the following formula:

$$\%\text{CV} = (\text{Standard deviation}/\text{Mean}) \times 100.$$

RESULTS

Standardization of RIA for Mithun Plasma Progesterone Determination

Titration of Progesterone Antisera

Serial dilution of antiserum from 1 : 500 to 1 : 25,600 was made to estimate the dilution of the antiserum to be used by the direct radioimmunoassay method. An antibody titre of 1 : 8000 was found to be most suitable and achieved a count of around 1500 at maximum binding tubes.

Assay Validation

Assay Interference and Sensitivity

To determine the possible interference of plasma with assay sensitivity, progesterone standards ranging from 2 to 500 pg/20 μL/tube, in various amounts of plasma (0, 10, and 20 μL) were run in the assay. There was no difference in the absolute binding sensitivity between 10 and 20 μL plasma volumes, which was similar to that which was observed with buffer standards; however, a slight decrease in sensitivity was seen when standard taken in 20 μL plasma

was run in the assay. Increasing plasma volumes also showed a slight reduction in the final counts (Figures 1A and 1B). Keeping all these aspects in view, standards were subsequently prepared in charcoal-treated hormone-free plasma and run along with unknowns in the test. The counts for non-specific binding tubes, using both the volumes of plasma, were low, ranging from 93 to 112. All assays were, hence, conducted with 20 μL of unknown plasma samples and standards per tube, in duplicate, to increase the sensitivity of the assay. The lowest progesterone detection limit, significantly different from zero concentration, was 2 pg/tube/20 μL plasma, which corresponds

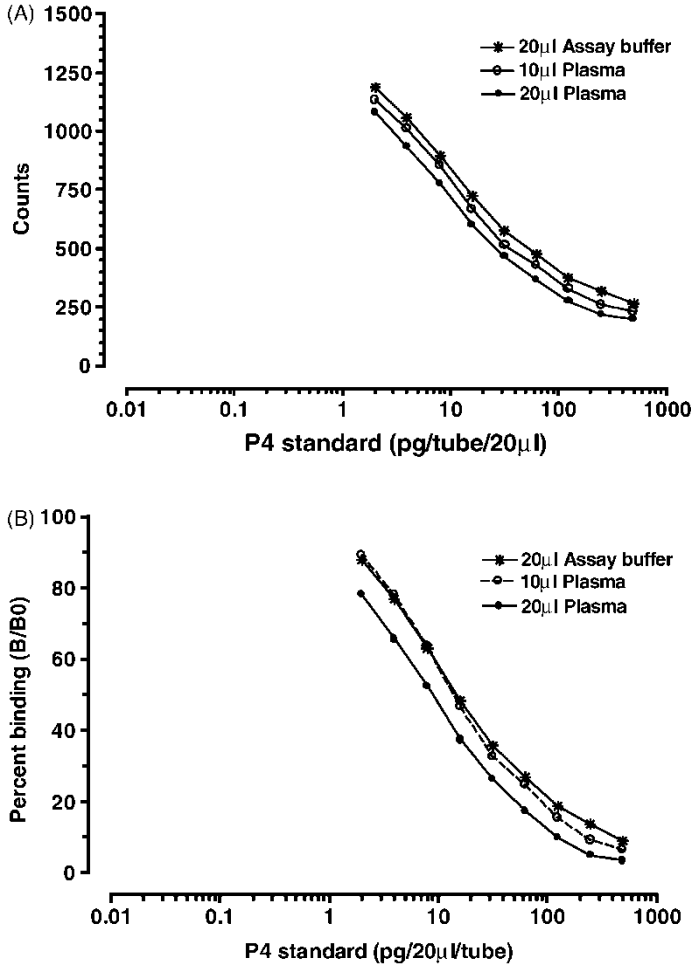

Figure 1. Influence of different volumes, viz., 0, 10, and 20 μL of mithun plasma on counts displacement (A) and on percent binding (B) in progesterone (P4) standard curve. Counts were taken using a liquid scintillation counter.

to 0.1 ng/mL plasma. The 50% relative binding (B/B_0) sensitivity was 32 pg/20 μL plasma/tube, which corresponded to 1.6 ng/mL plasma.

Intra- and Inter-Assay Precision

Intra- and inter-assay coefficients of variation, determined using pooled plasma containing 0.8 ng/mL and 12.5 ng/mL in assays, were 7.23, 9.56, and 6.13, 10.12 percent, respectively.

Biological Validation

Mean progesterone concentrations in blood samples collected from three cyclic mithuns is presented in Figure 2. The progesterone level reached a peak level at around day 14 of the estrous cycle (day 21 of sampling) and, at a day around estrus, the level was found to be the lowest, 0.35 ng/mL.

The plasma progesterone concentrations estimated in the blood samples collected weekly from three early pregnant mithuns is presented for individual mithun in Figure 3A. In animal No. 1, the plasma progesterone concentration, which was recorded to be 2.5 ng/mL at day 0 of sampling, increased gradually as the pregnancy advanced and reached a level of 6.6 ng/mL at day 42 of sampling. A similar trend of plasma progesterone concentration was recorded in animal No. 2, where the values were 3.7 and 7.9 ng/mL at day 0 and 42 of sampling, respectively. The animal No. 3 registered plasma progesterone concentration of 1.5 ng/mL at day 0 of sampling, which increased gradually and reached a level of 4.7 ng/mL at day 42 of sampling.

The plasma progesterone concentrations estimated in the blood samples collected weekly from two late pregnant mithuns is presented for individual

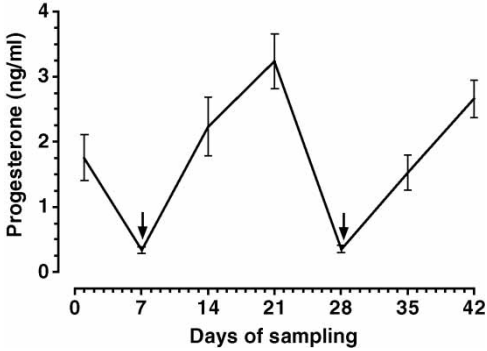

Figure 2. Progesterone (mean ± SEM) profile in cyclic mithuns (n = 3). Blood samples were collected weekly from all three cyclic mithuns for 42-day period. Arrows indicate day at estrus.

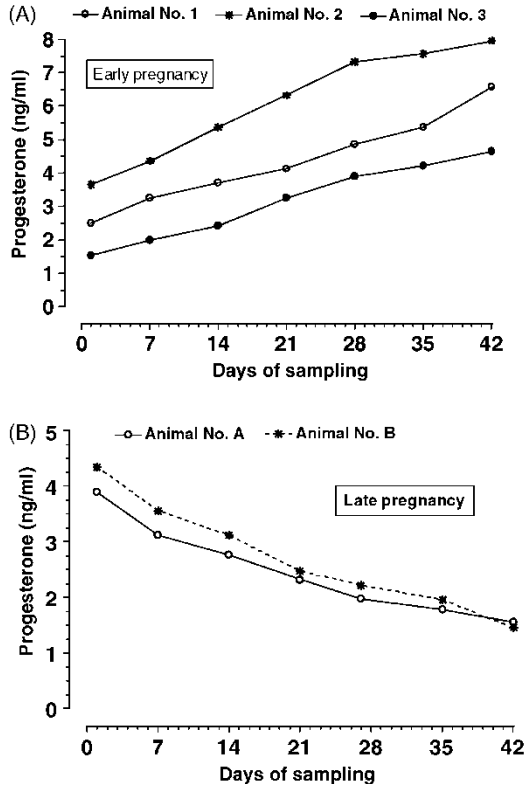

Figure 3. Progesterone profiles for individual pregnant mithuns during early (A; n = 3) and late pregnancy (B; n = 2). Blood samples were collected weekly from all mithuns for 42-day period.

mithun in Figure 3B. In animal No. A, the level of plasma progesterone was estimated to be 3.9 ng/mL at day 0 of sampling, which decreased gradually and reached a level of 1.5 ng/mL at day 42 of collection. A similar change of plasma progesterone was recorded in animal No. B, which exhibited 4.4 ng/mL of plasma progesterone at day 0 with the level declining gradually as the pregnancy advanced, to register 1.4 ng/mL at day 42 of sampling.

DISCUSSION

To the best of our knowledge, the method described here is the first report for estimation of progesterone in mithun plasma. The results of our investigations show that measurement of progesterone in unextracted mithun plasma by the RIA method presented here is sufficiently reliable and simple. One of the major advantages in our RIA is that it avoids a troublesome and

time-consuming extraction procedure. Previously, progesterone was estimated using a preliminary solvent extraction step (extraction RIA) in ruminants.[16–19] The plasma volume required for extraction of RIA was also higher, i.e., 100 to 500 μL.[16–19] In our RIA, there was a decrease in counts with increasing plasma volumes, although the sensitivities and the relative binding percentage did not change when 10 and 20 μL plasma samples were taken along with standards (Figure 1A and B). In order to compensate for the plasma volume interference effect, it is necessary to use the same plasma volumes for standards and for unknowns. A high sensitivity of 0.1 ng/mL was obtained when 20 μL of plasma was taken for estimation. The sensitivity of the present RIA is higher than those reported in other ruminants[16,20] and comparable to extraction RIA [17,21] and to direct RIA.[8,22] The level of plasma progesterone recorded in mithun cows during the estrous cycle is in accord with other bovines.[4,5,8] The similar trends of plasma progesterone level have been reported for early and late pregnant bovines, as reported herein in mithuns.[3,5,8,9,23] The difference among mithuns in plasma progesterone concentrations during early and late pregnancy periods, in the present study, was due to different days of start of sampling from commencement of pregnancy in different animals. The RIA procedure described herein was sufficient to determine the low physiological baseline progesterone concentrations at estrus and high plasma progesterone levels during pregnancy. Moreover, the use of unextracted plasma for the assay avoids the troublesome and laborious extraction procedure. In conclusion, the progesterone RIA described here is highly sensitive, reliable, and simple enough to estimate mithun progesterone of all physiological variations.

ABBREVIATIONS

RIA	Radioimmunoassay
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
PPO	2,5-diphenyloxazole
POPOP	1,4-dis-2-(5-phenyloxazolyl) benzene

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